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MYC2 Differentially Modulates Diverse Jasmonate-Dependent Functions in *Arabidopsis* [W]

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The *Arabidopsis thaliana* basic helix-loop-helix Leu zipper transcription factor (TF) MYC2/JIN1 differentially regulates jasmonate (JA)-responsive pathogen defense (e.g., *PDF1.2*) and wound response (e.g., *VSP*) genes. In this study, genome-wide transcriptional profiling of wild type and mutant *myc2/jin1* plants followed by functional analyses has revealed new roles for MYC2 in the modulation of diverse JA functions. We found that MYC2 negatively regulates Trp and Trp-derived secondary metabolism such as indole glucosinolate biosynthesis during JA signaling. Furthermore, MYC2 positively regulates JA-mediated resistance to insect pests, such as *Helicoverpa armigera*, and tolerance to oxidative stress, possibly via enhanced ascorbate redox cycling and flavonoid biosynthesis. Analyses of MYC2 *cis* binding elements and expression of MYC2-regulated genes in T-DNA insertion lines of a subset of MYC2–regulated TFs suggested that MYC2 might modulate JA responses via differential regulation of an intermediate spectrum of TFs with activating or repressing roles in JA signaling. MYC2 also negatively regulates its own expression, and this may be one of the mechanisms used in fine-tuning JA signaling. Overall, these results provide new insights into the function of MYC2 and the transcriptional coordination of the JA signaling pathway.

INTRODUCTION

In response to exogenous and endogenous cues, plants synthesize various fatty acid derivatives that act as signaling molecules. Among these, jasmonic acid and its volatile methyl ester, methyl jasmonate (MeJA), collectively known as jasmonates (JAs), are the best characterized fatty acid–derived cyclopentanone signals. JAs modulate a number of vital physiological processes, including defense against pathogens and insects, wound responses, secondary metabolite biosynthesis, and flower development and fertility (reviewed in Cheong and Choi, 2003).

Receptors for JAs have not been identified, but following the perception of JA, a number of cellular signaling processes occur that presumably result in the posttranslational modification (e.g., phosphorylation) of upstream regulatory proteins (Rojo et al., 1998), transcriptional activation of JA-responsive transcription factors (TFs), and downstream response genes. To date, forward genetic approaches have been instrumental in the identification of various genes involved in the JA signaling pathway (Berger, 2002). One of the first JA signaling mediators identified after map-based cloning of the mutated locus in the *coi1* mutant is the *CORONATINE-INSENSITIVE1* (*COI1*) gene, which encodes an F-box protein involved in the ubiquitin-proteasome pathway (Xie et al., 1998). Most JA-regulated responses, including fertility and defense against pests and pathogens, are altered in the *coi1* mutant, suggesting that COI1 acts relatively upstream in the JA signaling pathway (reviewed in Lorenzo and Solano, 2005). However, currently, very little is known about negative regulators of JA-responsive gene expression that might be ubiquitinated in a COI1-dependent manner. Histone deacetylases (HDACs), acting as transcriptional repressors of gene expression, have been implicated as potential COI1 targets. Indeed, COI1 interacts in planta with HDA6, which encodes a histone deacetylase in *Arabidopsis thaliana* (Devoto et al., 2002). Another HDAC involved in JA signaling is HDA19, which functions as a negative regulator of defense genes positively regulated by ETHYLENE RESPONSE FACTOR1 (ERF1) (Zhou et al., 2005), an important TF in JA- and ethylene (ET)-dependent signaling for pathogen defense (Lorenzo et al., 2003).

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MYC2, a basic helix-loop-helix (bHLH) domain–containing TF, acts as both activator and repressor of distinct JA-responsive gene expression in *Arabidopsis* (Lorenzo et al., 2004). *MYC2* is allelic to the *JAI1*/*JIN1* (for *JASMONATE-INSENSITIVE1*) locus, which was first identified in a mutant screen for reduced sensitivity of its roots to exogenous JA (Berger et al., 1996). MYC2 is also known as RD22BP1, RAP-1, or ZBF1 (Abe et al., 1997; de Pater et al., 1997; Yadav et al., 2005). Despite the potential significance of MYC2 as a major player in the JA signaling pathway (Lorenzo and Solano, 2005), only a few MYC2-regulated genes have been identified to date. These genes include the JA-responsive pathogen defense genes *PDF1.2*, *CHIB*/*PR3*, and *HEL*/*PR4* and are negatively regulated by MYC2 (Anderson et al., 2004; Lorenzo et al., 2004). Consequently, *myc2/jin1* mutant plants show increased resistance to fungal pathogens such as *Plectosphaerella cucumerina*, *Botrytis cinerea*, and *Fusarium oxysporum* (Anderson et al., 2004; Lorenzo et al., 2004) and the bacterial pathogen *Pseudomonas syringae* (Nickstadt et al., 2004; Laurie-Berry et al., 2006). In addition, MYC2 positively regulates the JA- and wound/insect-responsive genes *VSP*, *LOX*, and *TAT* (Boter et al., 2004; Lorenzo et al., 2004). However, it is currently unknown whether insect tolerance is compromised in *myc2/jin1*. Also unknown is whether MYC2 has additional roles in modulating other JA-regulated genes and plant functions.

The JA signaling pathway interacts extensively with other hormonal and developmental signaling pathways, and emerging evidence suggests that MYC2 plays a pivotal role in modulating some of these interactions. For instance, MYC2 also acts as a positive regulator of abscisic acid–dependent drought responses (Abe et al., 2003) and is required for the suppression of salicylic acid–dependent defenses during infection by *P. syringae* (Laurie-Berry et al., 2006). Interactions between JA and ET as well as JA and auxin signaling are also known (reviewed in Woodward and Bartel, 2005), but it is not known whether MYC2 has a role in regulating such interactions.

Here, we address the following two questions. (1) What other JA-dependent cellular and phenotypic responses, outside of disease resistance and wound response, are regulated by MYC2? (2) How does MYC2 modulate diverse JA responses at the transcriptional level? Using genome-wide gene expression analysis of MeJA-treated wild-type and *myc2/jin1* plants, we identified a large number of JA-responsive and MYC2-regulated genes, including a number of TF genes. In addition, comparative phenotypic and biochemical analyses of *myc2/jin1* wild-type and *myc2/jin1* plants constitutively expressing *MYC2* provided functional evidence that MYC2 positively regulates oxidative stress tolerance, flavonoid biosynthesis, and insect herbivory resistance and negatively regulates Trp metabolism, leading to the JA-dependent synthesis of defensive compounds such as indole glucosinolates (IGs). Furthermore, we show that JA activates auxin biosynthesis and that MYC2 is required for the inhibition of root elongation by auxin transport inhibitors. Finally, differential expression of diverse TF genes during JA signaling in the *myc2/jin1* mutant along with DNA binding and expression studies of T-DNA lines of MYC2-modulated TFs have led to the proposal that MYC2 probably acts through the transcriptional orchestration of other TFs, which in turn regulate downstream JA response genes involved in diverse JA-dependent plant processes.

RESULTS

MYC2 Modulates Gene Expression in a JA-Dependent Manner

A genome-wide transcript analysis was undertaken to identify the *Arabidopsis* genes that are regulated by MYC2. In three independent biological experiments, wild-type (Columbia [Col-0]) and *myc2/jin1* (*jin1-9*) (Anderson et al., 2004) plants were either treated with 0.1 μM MeJA for 6 h or mock-treated as a control. Whole-genome gene expression of the samples was analyzed using Affymetrix ATH1 GeneChips (for full experimental details, see Methods and Supplemental Methods online). Stringent statistical analysis of the data was performed by means of two-way ANOVA for the factors of genotype (Col-0 versus *jin1-9*) and JA treatment (mock versus 0.1 μM MeJA), and the results are summarized in Figure 1A. A complete list of genes that are significantly affected in their expression by either of these factors is also provided in Supplemental Table 1 online. The Venn diagram given in Figure 1A summarizes the ANOVA analysis. Most of the MYC2-modulated genes were also induced by MeJA in the wild-type background (Figure 1C). In addition, a substantial number of genes (Figure 1A) had significance ($P < 0.05$) for interaction between the genotype and treatment factors (see Supplemental Table 1 online). Overall, the data from our GeneChip experiments suggest that MYC2 probably modulates the expression of a significant portion of all *Arabidopsis* genes. Importantly, comparison of differentially expressed genes in *jin1-9* with their MeJA-responsive expression in the wild type revealed that the MYC2 dependence of gene expression was predominantly present under MeJA treatment (Figures 1B and 1C).

To confirm the outcome of the microarray analysis, three additional independent biologically replicated time course experiments were set up with Col-0 and *jin1-9* with or without MeJA treatment, and samples were harvested at 1, 3, 6, and 24 h after treatment. The expression from selected MYC2-regulated genes was determined by quantitative real-time PCR (Q-RT-PCR), and the results for genes discussed in some detail are shown in Figures 3A, 5A, and 6A below and in Supplemental Figure 1 online. For the majority of the tested genes, the Q-RT-PCR experiments confirmed the microarray results, with significantly altered expression in at least one time point.

The differentially expressed genes in *myc2/jin1* included known JA- and MYC2-regulated genes (e.g., *PDF1.2*, *CHI*/*PR3*, *HEL*/*PR4*, and *VSP*) as well as genes involved in a variety of other JA-regulated functions, such as Trp metabolism, phenylpropanoid and flavonoid metabolism, sulfur metabolism, oxidative stress tolerance, hormone biosynthesis, insect pest resistance, and senescence (see Supplemental Table 1 online). These results indicated that MYC2 regulates a wider array of JA responses than was previously known. In subsequent experiments (see below), we further investigated phenotypic responses providing a functional role for MYC2 in some of these JA-mediated processes.

MYC2 Negatively Regulates Trp Metabolism during JA Signaling

The microarray and Q-RT-PCR analyses showed that several genes involved in Trp biosynthesis and Trp-derived secondary

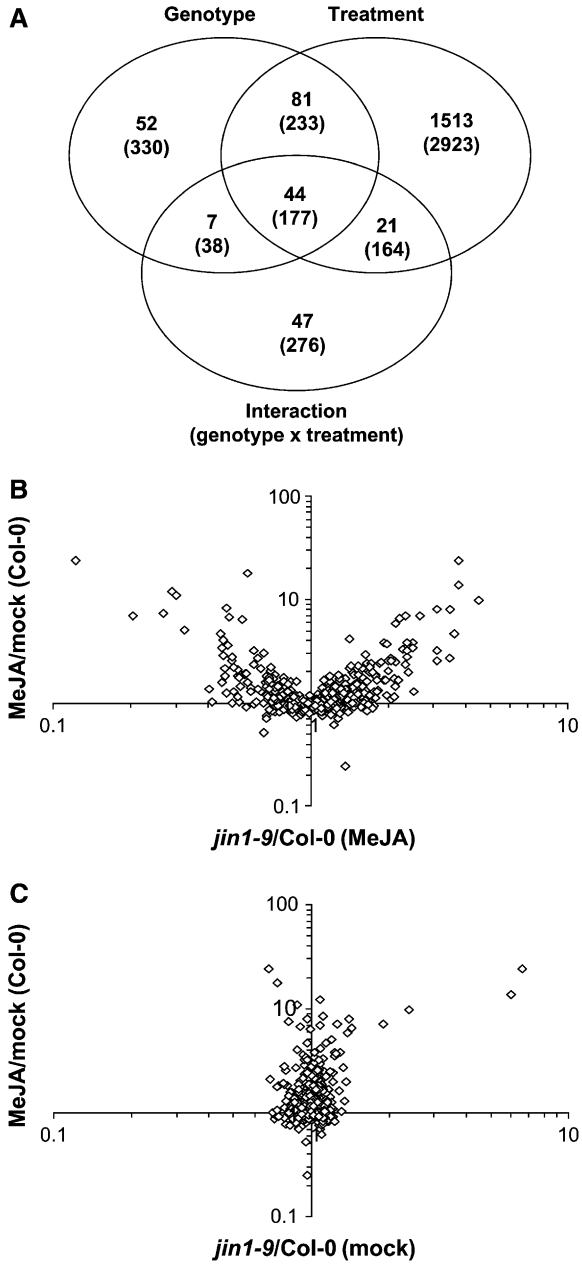

Figure 1. Summary of the Affymetrix GeneChip Experiment.

(A) Statistical analysis of the effect on gene expression of the factors genotype (Col-0 versus *jin1-9*) and treatment (mock versus 0.1 μM MeJA) by two-way ANOVA of the microarray expression data. The number of genes showing a significant change at $P < 0.01$ and $P < 0.05$ (in parentheses) is shown.

(B) and **(C)** Biplots of the ratios of expression values from the GeneChip experiments. Genes that are significant for genotype ($P < 0.05$) are shown as white diamonds (778 genes). Each data point is the ratio of the averages of three independent biological replicates. The *y* axes show the ratio of average expression levels of MeJA-treated wild-type plants (Col-0) over mock-treated wild-type plants. The *x* axes show the ratio of average expression levels of MeJA-treated *myc2* mutant (*jin1-9*) plants over MeJA-treated wild-type plants **(B)** and the ratio of average expression levels of mock-treated *myc2* mutant plants over mock-treated wild-type plants **(C)**.

metabolism were differentially expressed in response to MeJA in the *jin1-9* mutant compared with their expression in similarly treated wild-type plants (Figures 2A and 3A; see Supplemental Table 1 online). Trp biosynthesis genes (*ASB*, *IGPS*, *TSA1*, and *TSB2*), IG biosynthesis genes and their transcriptional regulators (*MYB51*, *ATR4*/*CYP83B1*/*SUR1*, *APS3*, *APR3*, and *ST5a*) and the camalexin biosynthesis gene *PAD3*/*CYP71B15* were consistently expressed at higher levels in *jin1-9* than in the wild type following MeJA treatment, suggesting that MYC2 acts as a negative regulator of the Trp metabolic pathway during JA signaling (Figure 2A; see Supplemental Table 1 online).

To determine whether the increased MeJA responsiveness of the Trp metabolism genes leads to alterations in the activity of the Trp metabolic pathway in the *myc2/jin1* mutant, a 5-methyl-DL-Trp (5MT) root growth inhibition assay was set up using the

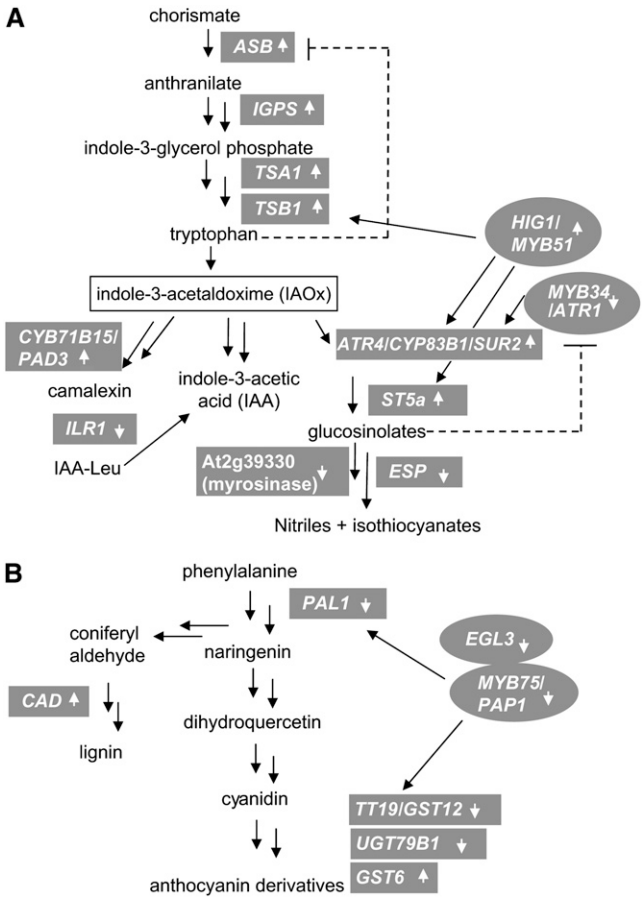

Figure 2. Schematic Summary of MYC2-Regulated Trp and Flavonoid Metabolism Genes.

MYC2-regulated Trp and Trp-derived secondary metabolism **(A)** and phenylpropoanoid and flavonoid metabolism **(B)** genes. Significant up-regulation and downregulation in the MeJA-treated *myc2/jin1* mutant relative to the similarly treated wild-type plants are indicated with up arrows and down arrows, respectively. Enzymes are depicted in rectangular boxes, and TFs are shown in elliptical boxes. The double arrows used between the substrates indicate multiple biochemical steps. See Supplemental Table 1 online for details of the genes.

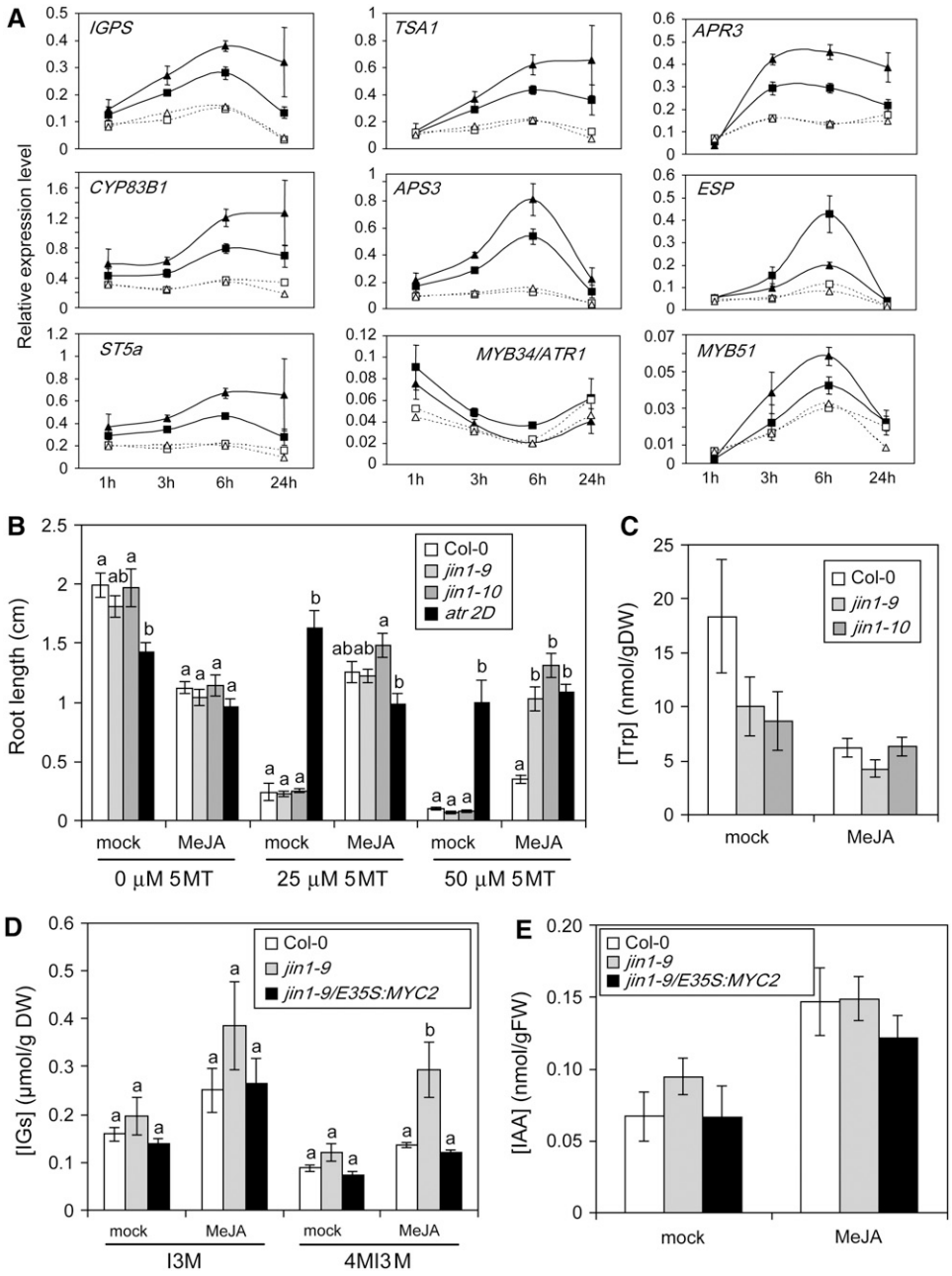

Figure 3. MYC2 Negatively Regulates Trp Metabolism in a JA-Dependent Manner.

(A) Q-RT-PCR expression analysis of Trp metabolism genes after treatment with 0.1 μM MeJA (black symbols) or mock treatment (white symbols) in Col-0 (squares) and *jin1-9* (triangles). Data are expressed as relative RNA levels ($[mRNA]_{gene}/[mRNA]_{actin}$) and are means of three biological replicates (>30 pooled plants each); error bars denote SE.

(B) Root lengths of MeJA-treated (0.5 μM) and 5MT-treated 7-d-old *Arabidopsis* seedlings. Values (representative of two independent experiments) are means of >20 seedlings for each treatment/genotype combination; error bars denote SE. Values annotated with different letters are significantly different ($P < 0.01$; Tukey's least significant difference [LSD]). Note that at the relatively low MeJA concentrations used, the difference in root length inhibition between the wild type and *jin1-9* is not significant.

(C) Soluble Trp levels of 5-week-old *Arabidopsis* plants treated with 0.1 μM MeJA for 24 h. Values are means of three biological replicates (>20 pooled plants each); errors bars denote SE.

(D) IG levels of 5-week-old *Arabidopsis* plants treated with 50 μM MeJA for 48 h. I3M, indolyl-3-methyl glucosinolate; 4MI3M, 4-methoxy-indolyl-3-methyl glucosinolate. Values are means of three biological replicates (>20 pooled plants each); errors bars denote SE. Values annotated with different letters are significantly different ($P < 0.01$; Tukey's LSD).

(E) Free indole acetic acid (IAA) levels of 5-week-old *Arabidopsis* plants treated with 50 μM MeJA for 48 h. Values are means of three biological replicates (>20 pooled plants each); errors bars denote SE.

jin1-9 and *jin1-10* mutants (Anderson et al., 2004) as well as the *atr2D* mutant (Smolen et al., 2002) as a control. The toxic Trp analog 5MT acts by triggering feedback inhibition of anthranilate synthase activity without substituting for the nutritional role of Trp (Bender and Fink, 1998) (Figure 2A). At least two classes of *Arabidopsis* mutants show 5MT resistance: mutants with feedback resistance mutations in the anthranilate synthase catalytic subunits and mutants with increased expression of Trp metabolism genes (Smolen et al., 2002). Our experiments revealed that 5MT was toxic to the wild type and the two *myc2/jin1* mutant lines at both 25 and 50 μM concentrations. Consistent with previous observations (Smolen et al., 2002), the control 5MT-resistant mutant *atr2D* was virtually unaffected by 25 μM 5MT (Figure 3B). Importantly, these assays showed that the 5MT toxicity leading to the inhibition of root elongation was reduced in the seedlings germinated in the presence of MeJA, suggesting that MeJA-mediated alterations in Trp metabolism genes can indeed lead to changes in the Trp pathway. We found that at 50 μM 5MT, MeJA treatment significantly enhanced resistance to 5MT in the *myc2/jin1* lines but not in the wild type (Figure 3B). MeJA-mediated 5MT resistance observed in *atr2D* at 50 μM 5MT was similar to that in *myc2/jin1* plants. These results indicate that the MeJA-mediated changes observed in the expression of Trp pathway genes have altered Trp metabolism to a greater degree in the *myc2/jin1* mutant than in the wild type, leading to a JA-dependent increase in 5MT resistance.

Interestingly, only the 5MT-resistant mutants with anthranilate synthase feedback resistance show increased soluble Trp levels, while mutants (e.g., *atr2D*) with increased expression of Trp metabolism genes show reduced Trp levels (Smolen et al., 2002). We thus measured soluble Trp levels in *jin1-9*, *jin1-10*, and wild-type plants after MeJA treatment. MeJA treatment resulted in reduced soluble Trp levels in all three lines ($P < 0.01$, two-way ANOVA) (Figure 3C). In addition, we found reduced soluble Trp levels in the *myc2/jin1* mutants relative to the wild type under mock conditions (Figure 3C). Together, these results are suggestive of an increased flux in the Trp pathway following MeJA treatment and in *myc2/jin1* mutants.

MYC2 Is a Negative Regulator of JA-Dependent IG Biosynthesis

One possible outcome of the increased activity of the Trp metabolic pathway would be on indole classes of secondary metabolites and the auxin hormone IAA synthesized through this pathway. Indeed, Trp is first converted into indole-3-acetaldoxime (IAOx) by the action of the cytochrome P450 enzymes CYP79B2 and CYP79B3. IAOx is then used in the biosynthesis of IGs, camalexin, and IAA (Figure 2A) (reviewed in Grubb and Abel, 2006). We noted that transcripts of *HIG1/MYB51*, encoding a positive regulator of the indole-glucosinolate biosynthesis genes (Gigolashvili et al., 2007), *ATR4/CYP83B1/SUR2*, encoding a cytochrome P450 monooxygenase that channels IAOx toward IGs (Barlier et al., 2000), and *ST5a*, encoding a sulfotransferase implicated in Trp-derived glucosinolate biosynthesis (Piotrowski et al., 2004), were more strongly induced by MeJA in *jin1-9* than in wild-type plants (Figures 2A and 3A; see Supplemental Table 1 online). By contrast, the glucosinolate catabolism gene *EPITHIOSPECIFIER PROTEIN* (*ESP*), encoding the ESP, and At2g39330, encoding a myrosinase putatively involved in glucosinolate breakdown, were significantly less induced by MeJA in *jin1-9* than in the wild type (Figures 2A and 3A; see Supplemental Table 1 online). To determine whether differential expression of these genes leads to altered IG levels, we measured the levels of three IGs in MeJA-treated and untreated plants of *jin1-9*, wild type, and *jin1-9* transformed with the *E35S:MYC2* construct (*jin1-9/E35S:MYC2*). These assays showed that the concentrations of two IGs, indolyl-3-methyl glucosinolate and 4-methoxy-indolyl-3-methyl glucosinolate, were MeJA-responsive ($P < 0.01$, two-way ANOVA); the latter was significantly higher in MeJA-treated *jin1-9* than in wild-type and *jin1-9/E35S:MYC2* plants (Figure 3D), while the levels of 1-methoxy-indolyl-3-methyl glucosinolate remained unchanged (data not shown).

We also found that the MeJA-responsive expression of *MYB34/ATR1* encoding a positive regulator of Trp and Trp-derived secondary metabolism genes was positively regulated by MYC2 (Figures 2A and 3A; see Supplemental Table 1 online). Although this seems to be contradictory in light of the actual increases observed at the IGs in the *myc2/jin1* mutant, transcription of *MYB34/ATR1* is negatively regulated by IGs in a negative feedback loop (Smolen and Bender, 2002; Celenza et al., 2005); thus, the reduced induction of *MYB34/ATR1* by MeJA in the *myc2/jin1-9* background could indeed be consistent with higher IG levels found in this mutant. Overall, these results suggest that MYC2 is a negative regulator of the JA-dependent biosynthesis of Trp-derived IGs in *Arabidopsis*.

In addition to IGs, the antimicrobial metabolite camalexin is derived from IAOx (Figure 2A). The gene *PAD3/CYP71B15*, which encodes a cytochrome P450 enzyme catalyzing the final step in camalexin synthesis (Schuhegger et al., 2006), was differentially expressed in the *jin1-9* mutant following MeJA treatment (Figure 2A; see Supplemental Table 1 online). Although we did not determine camalexin levels in the MeJA-treated *jin1-9* mutant, evidence from other studies shows that the increased expression of *PAD3* is associated with increased levels of camalexin (Zhou et al., 1999), while the *pad3* mutant displays camalexin deficiency (Thomma et al., 1999). Camalexin is known to be required in defense against necrotrophic fungal pathogens (Thomma et al., 1999), and the possible increase of the levels of this phytoalexin in the *myc2/jin1* mutant might contribute to the increased fungal disease resistance observed previously in this mutant (Anderson et al., 2004; Lorenzo et al., 2004).

MeJA-Induced Auxin Biosynthesis in *Arabidopsis*

We hypothesized that the increased flux in the Trp-metabolic pathway may also lead to alterations in IAA levels in MeJA-treated plants. Differential expression of *ATR4/CYP83B1/SUR1* in the *myc2/jin1* mutant following MeJA treatment and the apparent increase in IG levels observed here suggest that the increased flux might be directed toward IGs and that this might occur at the expense of IAA (Grubb and Abel, 2006). However, because several genes involved in Trp biosynthesis were expressed at higher levels in response to MeJA in the *myc2/jin1* mutant than in the wild type (Figure 2A), it is possible that IAOx levels were also increased in the *myc2/jin1* mutant. This could

lead to overall increases in IAA levels in MeJA-treated *myc2/jin1* plants relative to those in wild-type plants. In addition, Trp- and IAOx-independent but IGPS (for indole-3-glycerol phosphate synthase)-dependent IAA synthesis has been described (Ouyang et al., 2000), and *IGPS* was also differentially expressed in *jin1-9* following MeJA treatment (Figures 2A and 3A; see Supplemental Table 1 online). Therefore, we determined free IAA levels in MeJA-treated and -untreated plants of *jin1-9*, wild type, and *jin1-9/E35S:MYC2*. We found significantly increased levels of IAA ($P < 0.01$, two-way ANOVA) (Figure 3E) in MeJA-treated plants of all three lines relative to those in untreated plants, and this is consistent with MeJA's stimulatory effects on flux in the Trp metabolic pathway. However, these assays did not reveal any discernible difference in free IAA levels between the different genotypes assayed (Figure 3E).

It should be noted, however, that in *Arabidopsis*, IAA can also be synthesized through alternative pathways (reviewed in Woodward and Bartel, 2005). Our gene expression assays showed that the MeJA-responsive expression of *ILR1*, encoding an IAA-amino hydrolyase that releases free IAA from the conjugated forms (Bartel and Fink, 1995), was reduced in *jin1-9* relative to wild-type plants (Figure 2A; see Supplemental Table 1 online). Although the relative contribution of Trp-derived and ILR1-mediated IAA biosynthesis to final IAA levels in plant tissue is not known, it is possible that the potential increase in IAA levels in *myc2/jin1* through the activation of the Trp pathway might be negated by the reduced expression of *ILR1*. Nevertheless, the increased IAA level in MeJA-treated plants is a new finding and could contribute to JA-mediated growth regulation.

MYC2 Is a Positive Regulator of JA-Mediated Flavonoid Biosynthesis

MYC2, also known as RAP-1 (for R-homologous *Arabidopsis* Protein-1), shows significant sequence similarity to R proteins, regulating anthocyanin biosynthesis in maize (*Zea mays*) (de Pater et al., 1997). Indeed, the development of anthocyanin in *myc2/jin1* seedlings germinated in the presence of JA is abolished, while strong anthocyanin pigmentation develops in JA-germinated seedlings of *MYC2*-overexpressing plants (Lorenzo et al., 2004; this study; data not shown). Furthermore, coronatine (a JA analog and a phytotoxin produced by the bacterial pathogen *P. syringae*)–induced anthocyanin content was found to be reduced significantly in *myc2/jin1* plants (Laurie-Berry et al., 2006). Together, these results demonstrate that MYC2 is a positive regulator of the JA-mediated anthocyanin biosynthesis in *Arabidopsis*, although the molecular mechanism behind this observation is unknown.

Our large-scale gene expression analyses revealed that the MeJA responsiveness of several genes involved in flavonoid biosynthesis was reduced in the *jin1-9* mutant relative to that in wild-type plants (see Supplemental Figure 1 and Supplemental Table 1 online). Among these are *MYB75*/*PAP1* and *EGL3*, both encoding positive regulators of flavonoid biosynthesis (Borevitz et al., 2000; Zhang et al., 2003; Teng et al., 2005; Tohge et al., 2005). In addition, the genes positively regulated by MYB75/PAP1, such as *PAL1*, *TT19*/*GST12*, and *UGT79B* (Tohge et al., 2005), with well-studied roles in flavonoid biosynthesis (Kitamura et al., 2004; Rohde et al., 2004), showed differential expression in the *jin1-9* mutant (Figures 2A and 5A; see Supplemental Figure 1 and Supplemental Table 1 online). These results suggest that MYC2, possibly acting by modulating the expression of the positive regulators, MYB75/PAP1 and EGL3, positively regulates flavonoid biosynthesis in *Arabidopsis* during JA signaling. Interestingly, we found that the *CAD* gene involved in lignin biosynthesis showed increased expression in the mutant. This is consistent with the recent finding that a negative correlation exists between flavonoids and lignin biosynthesis through the phenylpropanoid pathway in *Arabidopsis* (Besseau et al., 2007).

MYC2 Is Required for the Sensitivity of Root Elongation to Auxin Transport Inhibitors

Accumulating evidence suggests that flavonoids (e.g., anthocyanins) act as negative regulators of auxin transport (Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004; Wasson et al., 2006; Besseau et al., 2007). Auxin transport is required for primary root elongation (Muday and Haworth, 1994; Jensen et al., 1998) and plant growth (Besseau et al., 2007). Alterations in flavonoid levels also modulate expression from genes encoding auxin transporters (Lazar and Goodman, 2006). Our transcript profiling experiments indicated that the JA-responsive expression of a putative auxin efflux carrier family protein (At1g76520) was increased in *jin1-9* relative to wild-type plants (see Supplemental Table 1 online). It is possible that the deficiency in MeJA-mediated flavonoid synthesis in *myc2/jin1* could alter auxin transport and consequently lead to the reduced inhibition of primary root elongation in plants grown in the presence of exogenous JA.

Naringenin, an early precursor in the flavonoid biosynthetic pathway, inhibits primary root elongation as a result of its inhibitory effects on auxin transport (Brown et al., 2001). Naringenin also complements the increased auxin transport phenotype of *tt4* mutant plants with reduced flavonoids and auxin transport (Brown et al., 2001). To determine whether MYC2 is required for root naringenin sensitivity, we germinated seeds of *jin1-9*, the wild type, and *jin1-9/E35S:MYC2* in the presence of naringenin and measured the primary root lengths (Figure 4A). The roots of *jin1-9/E35S:MYC2* were shorter than those of *jin1-9* and the wild type in the absence of naringenin. Although primary root elongation was inhibited in all lines by naringenin and the combination of naringenin and MeJA, *jin1-9/E35S:MYC2* roots were hypersensitive (as shown by the percentages in Figure 4A) to naringenin, MeJA, and the combination of both. We also tested the sensitivity of *jin1-9*, wild-type, and *jin1-9/E35S:MYC2* roots to 2,3,5-triiodobenzoic acid (TIBA), a synthetic auxin transport inhibitor. Again, the *jin1-9/E35S:MYC2* roots were hypersensitive (as shown by the percentages in Figure 4B) to TIBA in both the presence and absence of exogenous MeJA. These results suggest that MYC2 makes primary root elongation more sensitive to inhibition by natural and synthetic auxin transport inhibitors and therefore might have a role in regulating auxin transport. We speculate that this effect of MYC2 might be due to MYC2's positive regulatory effects on JA-mediated flavonoid synthesis.

To further test the hypothesis that alterations in flavonoid levels influence root elongation in the presence of JA, we examined the sensitivity of *max1* roots to MeJA. This mutant was selected because, similar to MYC2, the *MAX1*/*CYP71B1* gene product acts

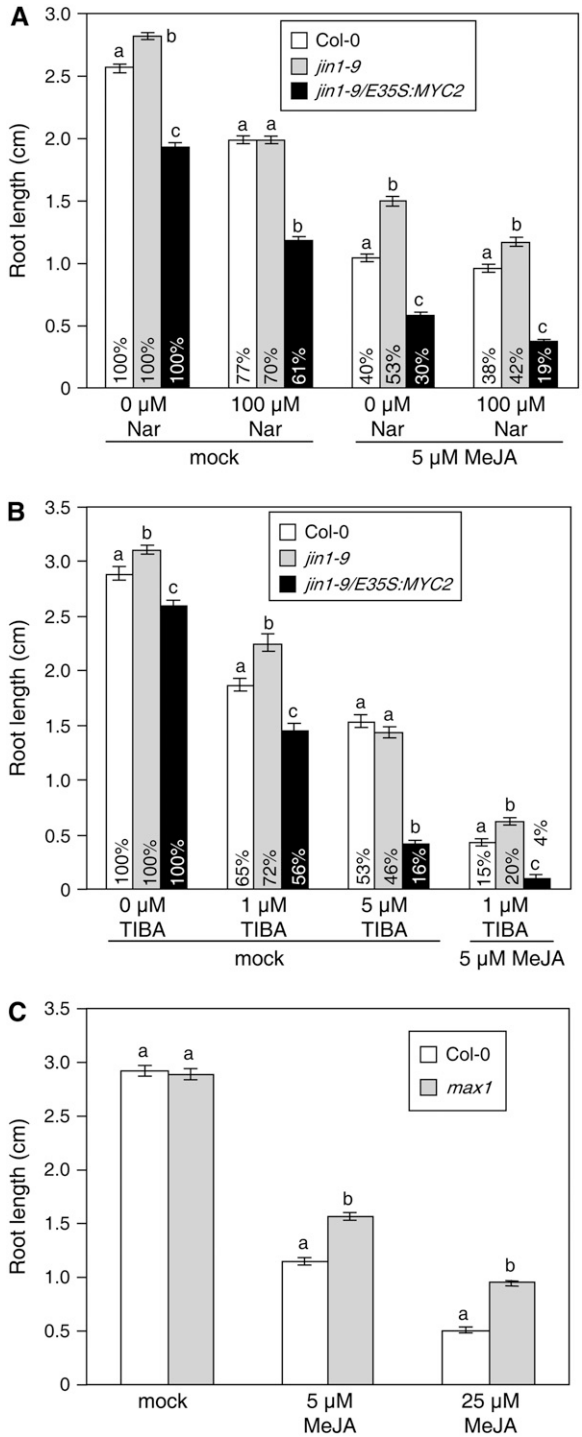

Figure 4. MYC2 Is Required for Increased Sensitivity of Root Elongation to Natural and Synthetic Auxin Transport Inhibitors.

(A) Root lengths of MeJA-treated and naringenin-treated (Nar) 10-d-old wild-type, *jin1-9*, and *jin1-9/E35S:MYC2 Arabidopsis* seedlings. Values (representative of two independent experiments) are means of >30 seedlings for each treatment/genotype combination; error bars denote SE. Values annotated with different letters are significantly different ($P < 0.01$; Tukey's LSD). Percentages of root lengths of the different lines are relative to the respective untreated controls.

(B) Root lengths of MeJA- and TIBA-treated 10-d-old wild-type, *jin1-9*, and *jin1-9/E35S:MYC2 Arabidopsis* seedlings. Values (representative of two independent experiments) are means of >30 seedlings for each treatment/genotype combination; error bars denote SE. Values annotated with different letters are significantly different ($P < 0.01$; Tukey's LSD). Percentages of root lengths of the different lines are relative to the respective untreated controls.

(C) Root lengths of MeJA-treated 10-d-old wild-type and *max1 Arabidopsis* seedlings. Values (representative of two independent experiments) are means of >30 seedlings; error bars denote SE. Values annotated with different letters are significantly different ($P < 0.01$; Tukey's LSD).

as a positive regulator of flavonoid synthesis and as a negative regulator of auxin transport in *Arabidopsis* (Lazar and Goodman, 2006). Again, similar to MYC2, MAX1/CYP71B1 encoding a cytochrome P450 affects flavonoid biosynthesis by modulating the expression of the positive regulator *MYB75/PAP1*. As shown in Figure 4C, we observed significantly reduced sensitivity of *max1* roots to exogenously supplied MeJA, further suggesting a link between MeJA-mediated flavonoid synthesis and auxin transport that affects primary root elongation in *Arabidopsis*.

MYC2 Is Required for Oxidative Stress Tolerance

A link between JA and ascorbate biosynthesis and redox cycling has been established (Sasaki-Sekimoto et al., 2005; Wolucka et al., 2005). JA induces the expression of certain ascorbate biosynthesis genes and the genes encoding (mono) dehydroascorbate reductase (*MDHAR* and *DHAR*) involved in redox cycling. Furthermore, treatment with JA or MeJA increases the de novo synthesis of ascorbate together with DHAR and ascorbate peroxidase activity (Sasaki-Sekimoto et al., 2005; Wolucka et al., 2005). Together with anthocyanins, ascorbate is known to be the main reactive oxygen species (ROS) scavenger in plants (Nagata et al., 2003). We noted that the MeJA inducibility of genes involved in oxidative stress tolerance was reduced in *jin1-9* relative to the wild type (Figure 5A; see Supplemental Table 1 online). In addition, *TAT3*, encoding a Tyr aminotransferase that catalyzes the first step in the tocopherol (vitamin E) biosynthetic pathway (Sandorf and Hollander-Czytko, 2002), showed reduced MeJA responsiveness in *jin1-9* (Figure 5A; see Supplemental Table 1 online). Tocopherol is a JA- and stress-induced chloroplast-located antioxidant that neutralizes photosynthesis-derived ROS (reviewed in Munne-Bosch, 2005).

To determine whether MYC2 has a role in regulating oxidative stress defenses during JA signaling, we treated wild-type, *jin1-9*, and *jin1-9/E35S:MYC2* lines with the superoxide generator methyl viologen (Paraquat) after a pretreatment by MeJA for 6 h. Five days after methyl viologen treatment, 90% of *jin1-9* plants were dead (Figure 5B). By contrast, only 5 and 15% of treated *jin1-9/E35S:MYC2* and wild-type plants, respectively, were dead at this stage. The leaves of the majority of the wild-type and *jin1-9/E35S:MYC2* plants remained green (Figure 5B), and these plants subsequently recovered and produced seed. In the absence of prior MeJA treatment, no differential ROS

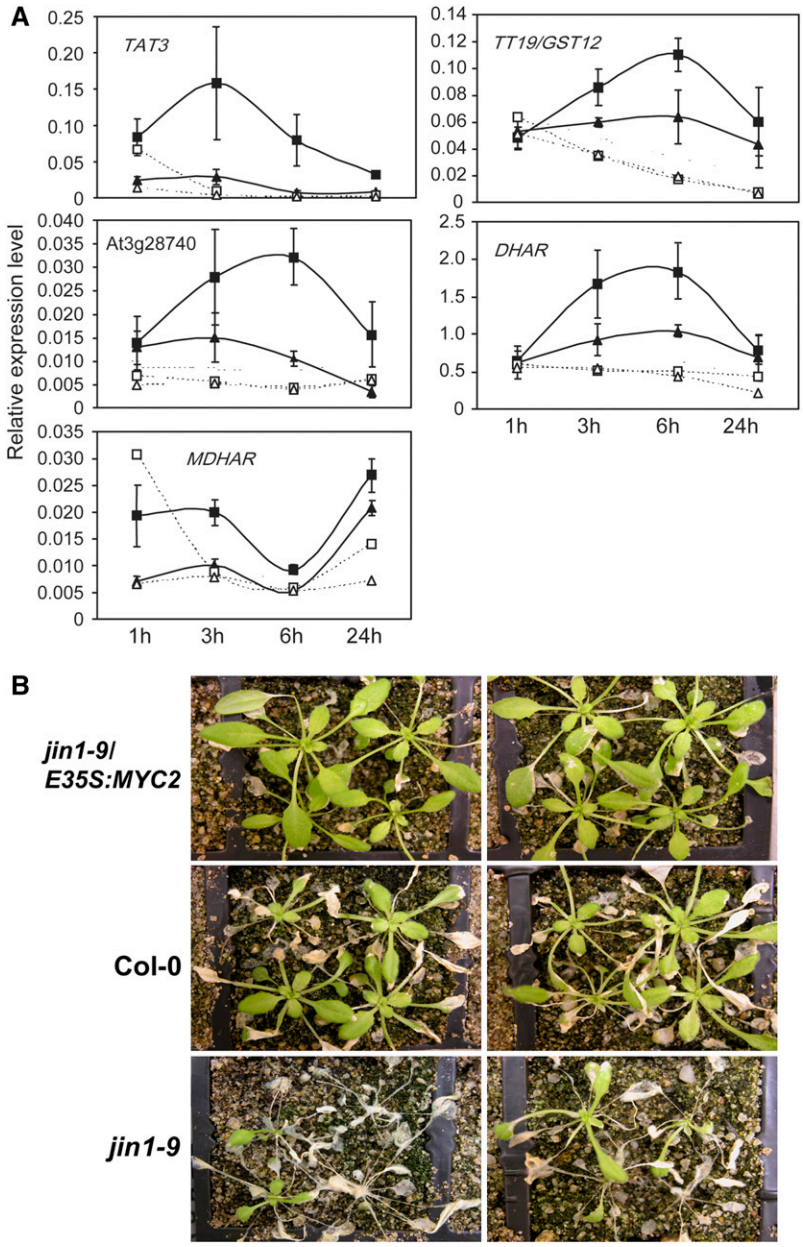

Figure 5. MYC2 Positively Regulates Oxidative Stress Tolerance in a JA-Dependent Manner.

(A) Q-RT-PCR expression analysis of anthocyanin- and ascorbate-related genes. See Figure 3A legend for details of Q-RT-PCR.
(B) *Arabidopsis* plant phenotypes at 4 d after treatment with 50 μM methyl viologen. Plants were pretreated for 6 h with 0.1 μM MeJA. Photographs are representative of four independent experiments each with 20 plants per genotype/treatment combination.

tolerance could be observed between the mutant and wild-type plants (data not shown). Overall, these results are consistent with the observation that MYC2 is a positive regulator of a subset of JA-responsive genes involved in oxidative stress protection.

MYC2 Positively Regulates Resistance to Insect Herbivory

The JA signaling pathway is known to regulate many inducible defenses effective against insects (for references, see Reymond et al., 2004). To date, no transcriptional regulator of the JA signaling pathway has been shown to alter insect tolerance in *Arabidopsis*. *MYC2*, a gene that is responsive to insect herbivory (Reymond et al., 2004), is a positive regulator of wound-responsive genes such as *VSP1*, *JR1*, *TAT*, and *LOX* (Boter et al., 2004; Lorenzo et al., 2004) that are also responsive to insect feeding. Here, we identified additional JA- and insect-responsive genes positively regulated by MYC2 (Figure 6A; see Supplemental Table 1 online). At least two of these genes with reduced MeJA

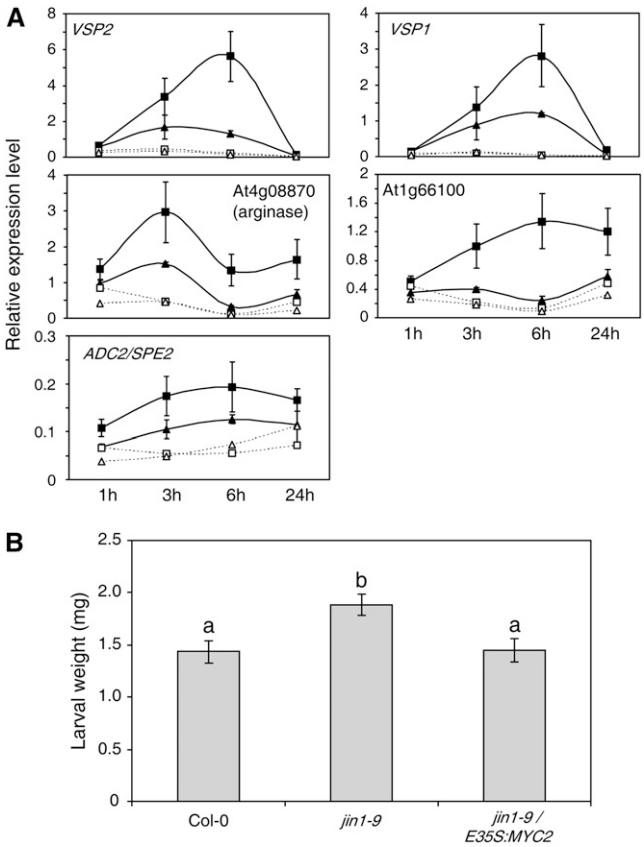

Figure 6. MYC2 Positively Regulates Resistance to *H. armigera* Herbivory during JA Signaling.

(A) Q-RT-PCR expression analysis of insect resistance and wound response genes. See Figure 3A legend for details of Q-RT-PCR.
(B) Average weight of *H. armigera* larvae at 6 d after neonate larvae were placed on 5-week-old *Arabidopsis* plants. Plants were pretreated for 24 h with 0.5 μM MeJA. Data are means of 15 individual plants challenged with five neonate larvae each; error bars denote SE. Values annotated with different letters are significantly different ($P < 0.01$; Tukey's LSD).

responsiveness in the *jin1-9* mutant have demonstrated anti-insect activities. *VSP2* encodes an anti-insect acid phosphatase enzyme (Liu et al., 2005), and the At4g08870 locus encodes an ortholog of the tomato (*Solanum lycopersicum*) arginase that reduces larval weight gain by degrading the essential amino acid Arg in the herbivore midgut (Chen et al., 2005). The reduced expression of these genes in the *myc2/jin1* mutant suggests that the insect resistance might be reduced in the mutant. Interestingly, however, as we reported above, we found increased levels of IGs in the *myc2/jin1* mutant (Figure 3D). This might suggest otherwise—that is, the *myc2/jin1* mutant may be more tolerant of insects, as IGs are often implicated in insect defense (Wittstock and Halkier, 2002). Therefore, we investigated whether insect herbivory is altered in the *myc2/jin1* mutants. No-choice feeding experiments were set up with the generalist herbivore *Helicoverpa armigera* (cotton bollworm or tobacco budworm). We found that the weight gain of neonate larvae feeding on MeJA-pretreated *jin1-9* plants was significantly higher than that on similarly treated wild-type and *jin1-9/E35S:MYC2* plants after 6 d of feeding (Figure 6B). These results show that MYC2 function is required for JA-mediated tolerance to *H. armigera* in *Arabidopsis*.

MYC2 Preferentially Binds to an Extended G-Box Motif

The large number of genes that displayed MYC2 dependence for their MeJA-responsive expression prompted us to quantitatively determine the optimal DNA binding site of MYC2. Determination of the optimal binding site can be of value to identify genes that may be regulated by MYC2 at the transcription level, thus making it possible to construct the potential MYC2 regulon. Previous reports indicated that MYC2 can bind to the G-box–related hexamers 5′-CACNTG-3′ (de Pater et al., 1997), 5′-CACATG-3′ (Abe et al., 1997), and 5′-(T/C)ACGTG-3′ (Yadav et al., 2005). However, these binding sites were determined in a nonquantitative and biased way using selected specific DNA sequences. Here, we opted for an unbiased and quantitative method (Xue, 2005) to identify the preferred DNA binding sites of MYC2.

Briefly, a purified MYC2-CelD-6xHis fusion protein was used for sequential steps of affinity selection of binding sequences from a pool of biotinylated random sequence oligonucleotides (30-mers) (Xue, 2005). The 6xHis-tagged cellulase (CelD) allows for affinity purifications (on cellulose or Ni) and quantification of the binding of selected oligomers to the MYC2 fusion protein (CelD as an enzymatic reporter). After the third and fourth selection rounds in the purification process, a massive increase in DNA binding activity, indicative of a strong enrichment for MYC2 binding sites in the oligonucleotide pools, was observed (data not shown). The oligonucleotides from the third and fourth selection round were cloned, and 40 clones from each pool were sequenced. Overall, the majority of these clones contained at least one CACGTG palindromic hexamer (G-box), suggestive of the G-box being the preferred MYC2 core binding site (Figure 7A). Some of the sequenced oligonucleotides contained the G-box–related motifs 5′-CACATG-3′ and 5′-CACGTT-3′. In total, 38 of the sequenced clones were amplified by PCR with biotinylated primers, the products purified, and their MYC2 binding activity measured. In Figure 7A, these oligonucleotides are ranked according to their MYC2 binding capacity. By and large, oligomers containing the G-boxes had the strongest MYC2 binding capacity, followed by those with the 5′-CACATG-3′ and 5′-CACGTT-3′ motifs.

The core G-box is a sequence element present at least once in nearly 30% of the 5′ upstream regions of all *Arabidopsis* genes (data not shown). Given the abundance of this sequence as well as the presence of many other bHLH proteins that can potentially bind to this sequence, it is likely that not all G-box–containing genes are regulated by MYC2. Therefore, we further defined the optimal DNA binding sequence of MYC2. Alignment of the palindromic G-boxes revealed additional conserved bases in the sequences that flank the G-box hexamer (Figure 7B). To determine whether these conserved flanking sequences contribute to the MYC2 DNA binding capacity, synthetic oligonucleotides were obtained with mutations in these regions (Figure 7C). Remarkably, all mutations introduced into these flanking nucleotides reduced the MYC2 DNA binding capacity of the D27 oligonucleotide. Both the upstream (positions 1 to 3 in Figure 7B)

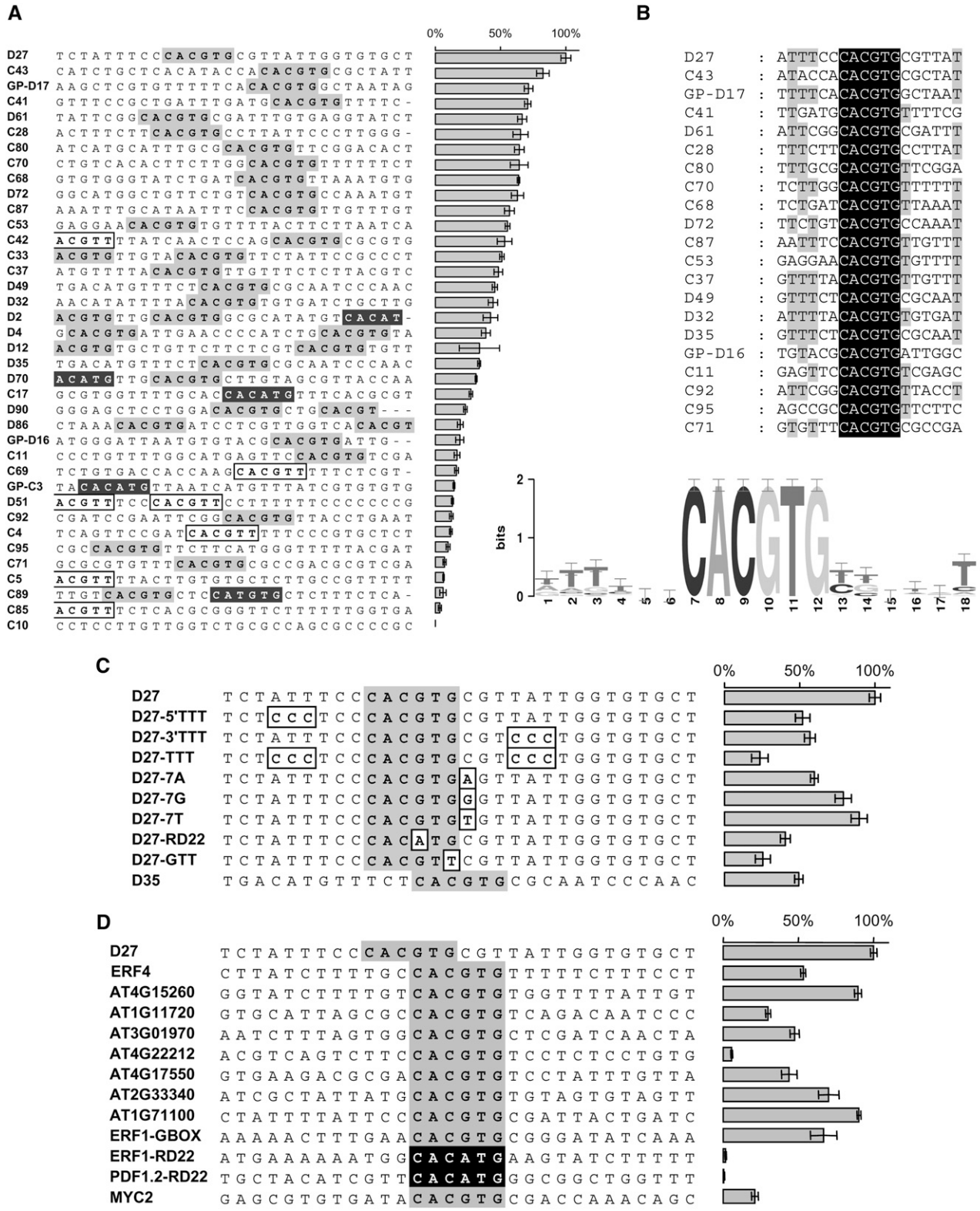

Figure 7. MYC2 Preferentially Binds to an Extended G-Box Motif.

(A) Sequences and MYC2 binding activities of 38 30-mers from affinity purification selection rounds 3 and 4. MYC2 binding activities for different sequences are expressed relative to the highest binding activity (relative binding activity) observed in D27. Values are means of three replicates; error

and downstream (positions 16 to 18 in Figure 7B) T-rich regions had a significant effect on the binding activity of MYC2. Mutation of the conserved pyrimidine at position 13 (Figure 7B) to a purine only slightly reduced the binding activity. More importantly, mutations introduced into the core G-box palindrome (5′-CACATG-3′ or 5′-CACGTT-3′) drastically diminished MYC2 binding activity (Figure 7C). A position–weight matrix based on the alignment shown in Figure 7B was then used in a stringent in silico screening for the presence of strong MYC2 binding sites in the 5′ upstream regions of *Arabidopsis* genes (see Supplemental Table 2 online). The screening revealed that the set of 778 MYC2-regulated genes is enriched for strong MYC2 binding sites compared with the whole *Arabidopsis* genome (8.0 versus 4.2%, respectively; $P < 0.01$, hypergeometric test). This enrichment was especially evident when MYC2-regulated TFs were compared with the whole *Arabidopsis* TF complement (20 versus 8.9%; $P < 0.01$) (see Supplemental Table 2 online). After clustering of the motifs in the upstream regions of the MYC2-regulated genes, 10 representative CACGTG core motifs were assessed for their MYC2 binding capacity (Figure 7D). These included the motifs found in the promoters of two MYC2-regulated TF genes (i.e., *ERF4* and *ERF1*) as well as in the promoter of *MYC2*. All of these *Arabidopsis* motifs displayed significant MYC2 binding activity except the one present in the upstream region of At4g22212.

Evidence That Negative Regulation of *PDF1.2* by MYC2 Is Mediated by Suppression of *ERF1*

Current models predict a direct mutual antagonism of MYC2 and ERF1 on the expression of pathogen defense response genes such as *PDF1.2* and *PR4*/*HEL* and wound response genes such as *VSP* and *LOX* (Lorenzo et al., 2004; Lorenzo and Solano, 2005). It was speculated that MYC2 might directly bind to the *PDF1.2* promoter to suppress its expression (Lorenzo et al., 2004). The *PDF1.2* promoter region contains a G-box–like motif, 5′-CACATG-3′ (Brown et al., 2003). This motif, depicted as *PDF1.2-RD22* in Figure 7D, is the same as the motif implicated as a MYC2 binding motif in the *RD22* promoter during abscisic acid– and drought-responsive expression of *RD22* (Abe et al., 1997). This G-box–like motif differs from the core of the optimal MYC2 binding site by at least one nucleotide (G). In our DNA binding experiments shown in Figure 7D, this motif and flanking sequences did not display any MYC2 binding capacity at all, suggesting that MYC2 might not interact directly with the *PDF1.2* promoter. Interestingly, the promoter region of *ERF1*, a gene involved in the ET- and JA-dependent induction of *PDF1.2* (Lorenzo et al., 2003), contains both a G-box (*ERF1*-GBOX) and a 5′-CACATG-3′ motif, depicted as *ERF1-RD22* in Figure 7D. We found that only the *ERF1*-GBOX had significant MYC2 binding affinity, most likely increased by the flanking T-rich regions and a C (pyrimidine) immediately downstream of the core hexamer (Figure 7D). These results, together with the increased MeJA responsiveness of *ERF1* (see Supplemental Figure 1 and Supplemental Table 1 online) in *myc2/jin1*, suggest that the negative regulation of *PDF1.2* expression by MYC2 is most likely mediated through the negative regulation of transcriptional activators of *PDF1.2* such as *ERF1*.

MYC2 Negatively Regulates Its Own Transcription

As shown in Figure 7D, the upstream region of the MYC2 gene contains a MYC2 binding site with a significant binding capacity, suggestive of a potential autoregulatory loop for MYC2 transcription. To investigate this possibility further, we comparatively analyzed *MYC2* transcript levels in the presence or absence of MeJA. In these experiments, we used a specific PCR primer pair (5′ untranslated region [UTR]) to distinguish the wild type and the mutant *MYC2* alleles from the transgenic allele in the complemented line (Figure 8B). Both the 5′UTR and the MYC2 primer pairs performed similarly for the wild type and the mutant *MYC2* alleles under both conditions tested. Also, *MYC2* expression, as detected by the MYC2 and 5′UTR primer pairs, was clearly induced by MeJA in both the wild type and *jin1-9*, while *MYC2* was constitutively expressed in *jin1-9/E-35S:MYC2* (Figure 8A). However, expression of the *MYC2* mutant allele detected using the 5′UTR primer pair was reduced significantly in the complemented line (*jin1-9/E-35S:MYC2*) compared with the mutant background, whereas the *E35S:MYC2* transgene, as detected by the MYC2 primer pair, remained highly expressed in the complemented background. This result suggests that MYC2 is capable of negatively regulating its own expression. To rule out any positional insertion effects of the transgene, several independent homozygous complemented lines were analyzed, and they all performed similarly (data not shown). In addition, we found that this negative regulation was not sensitive to cycloheximide (CHX) (Figure 8C), suggesting that new protein synthesis is not required for MYC2's negative regulatory effects on its own transcription. Given that multiple biotic and abiotic stress factors induce MYC2 expression, it is tempting to speculate that

Figure 7. (continued).

bars denote SD. Gray boxes, G-box; black boxes, 5′-CACATG-3′; white boxes, 5′-CACGTT-3′. Motifs at the edges of the 30-mers are completed by the sequences from the flanking regions of the random sequence oligonucleotide pool used for binding site selection (TAGC at the 5′ end and GCTG at the 3′ end; see Xue (2005) for complete sequences of flanking regions SP-A and SP-S1).

(B) Alignment of G-boxes and flanking sequences of MYC2-selected motifs containing a single CACGTG box with relative DNA binding activity of >30% of the highest affinity oligonucleotide (D27) (see **[A]**). Black boxes, 100% conserved; gray boxes, 75% conserved. The illustration depicting this alignment was created with WebLogo (Crooks et al., 2004).

(C) Sequences and MYC2 binding activities of D27-derived synthetic oligonucleotides. Binding activities of MYC2 and shading are as in **(A)**, except for the white boxes denoting mutations from the original D27 sequence.

(D) Sequences and MYC2 binding activities of motifs present in *Arabidopsis* promoter regions. Probes are synthetic oligonucleotides. The binding capacity of MYC2 is expressed as in **(A)**.

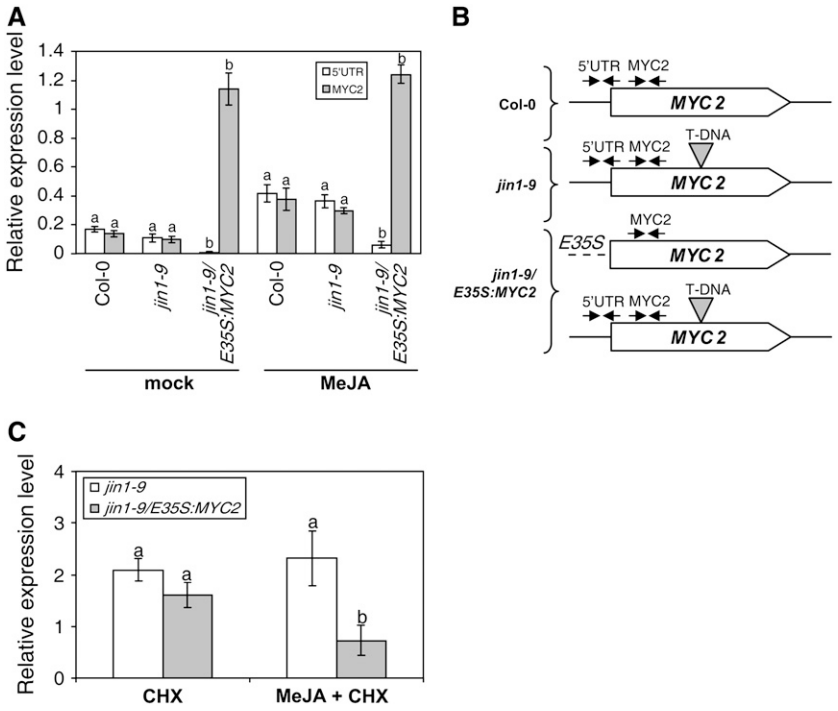

Figure 8. MYC2 Directly and Negatively Regulates Its Own Expression.

(A) Expression from the wild type, mutant, and transgenic *MYC2* alleles was comparatively examined using 5′UTR and MYC2 Q-RT-PCR primer pairs in mock- and 0.1 μM MeJA–treated plants of Col-0, *jin1-9*, and *jin1-9/E-35S:MYC2*. Note that as shown in **(B)**, the MYC2 primer pair binds to the mutant *MYC2* allele upstream from the T-DNA insertion site and detects similar transcript levels as found in the wild type. Error bars denote SE.
(B) Schematic illustration of the binding regions of the 5′UTR and MYC2 primers on the wild type, mutant, and both mutant and transgenic *MYC2* alleles on wild-type, *jin1-9*, and *jin1-9/E35S:MYC2* plants, respectively. Note that there is no 5′UTR binding site on the *E35S:MYC2* construct.
(C) Expression detected from the *jin1-9* and *E35S:MYC2* alleles by the MYC2 primer pair in CHX-treated and CHX- and MeJA-treated *jin1-9* and *jin1-9/E-35S:MYC2* plants. See text for details. Data are means of three biological replicates (more than five pooled plants each). Error bars denote SE. Values annotated with different letters in **(A)** and **(C)** are significantly different ($P < 0.01$; Tukey's LSD).

this negative autoregulation capability might be a mechanism that contributes to the fine-tuning of the signaling pathways by controlling MYC2 levels.

MYC2 Modulates the JA-Dependent Transcription of TF Genes

Many of the MYC2-regulated genes identified through transcript profiling and the subsequent Q-RT-PCR encode TFs (see Supplemental Figure 1 and Supplemental Table 1 online). A significant enrichment for strong MYC2 binding sites was found in the upstream regions of MYC2-regulated TF genes (see Supplemental Table 2 online). DNA binding assays shown in this report as well as in previous publications (de Pater et al., 1997; Abe et al., 2003; Boter et al., 2004; Yadav et al., 2005) clearly demonstrated that MYC2 can bind to the CACNGT core motif. Furthermore, our additional promoter analyses of a subset of MYC2-regulated TFs (given in Figure 10B) for the presence of CACGTG and CACATG motifs using the *Arabidopsis* Gene Regulatory Information Centre database (Palaniswamy et al., 2006) revealed that 82 and 88% of such TFs, respectively, had at least one of these core motifs in their promoters. Moreover, 71% of these TFs had at least one copy of both of these motifs in their promoters. Therefore, strong enrichment of these motifs in the promoters of MYC2-regulated TFs might suggest a hierarchical model in which MYC2 positively or negatively modulates the JA-dependent transcription of other TF genes, which in turn might control the JA-dependent transcription of the downstream JA response genes. In this model, MYC2 would be positioned relatively upstream in the JA signal transduction pathway, possibly downstream from COI1 (Lorenzo et al., 2004) and MKK3 and MPK6 mitogen-activated protein kinase pathways (Takahashi et al., 2007) but upstream from MYC2-regulated TFs. Significant functional overlaps observed for relatively large numbers of genes found to be differentially expressed in *myc2/jin1* (this study), *coi1* (Devoto et al., 2005), and *35S:ERF1* plants (Lorenzo et al., 2003) are consistent with this proposal.

To explore the possibility that MYC2 modulates JA-responsive gene expression through MYC2-dependent TFs, we obtained several homozygous T-DNA insertion lines for the following MYC2-regulated TF genes; *ERF2*, *ERF6*, *ERF11*, *WRKY26*, *WRKY33*, *MYB51*, *MYB109*, At1g33760, and *ZAT10*. An extensive Q-RT-PCR expression study was then set up to determine whether the expression of MYC2-regulated genes is affected in these mutant backgrounds in response to MeJA treatment. As shown in Figure 9A, the expression profiles of pathogen defense–related genes (cluster I) and wound response/insect

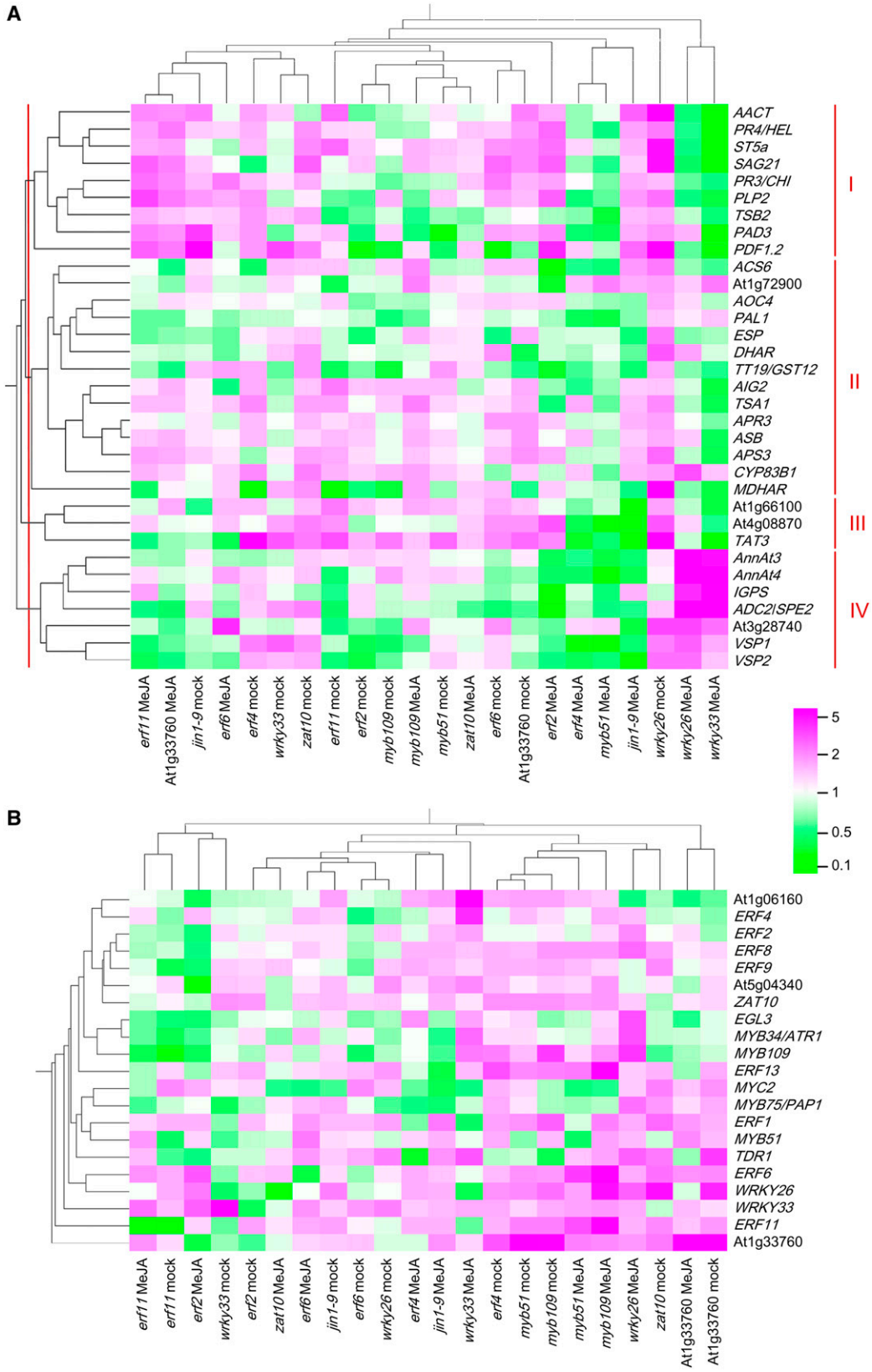

Figure 9. MYC2-Regulated TFs Modulate the Expression of MYC2-Regulated Genes.

resistance genes (clusters III and IV) suggest that these groups of genes are coregulated generally in an antagonistic manner. For instance, like ERF1 and in contrast with MYC2, WRKY33 acts as a negative regulator of wound response/insect resistance genes and as a positive regulator of pathogen defense–related genes during JA signaling. Similar to MYC2, ERF11 and At1g33760 downregulate pathogen defense–related genes and ERF2 activates wound response/insect resistance genes in JA-treated plants. MYB51 activates IGs biosynthesis genes in a JA-dependent manner. ZAT10, ERF4, and WRKY26 act as negative regulators of basal transcript levels of the majority of the genes in the absence of MeJA treatment. At least two of these TFs (ZAT10 and ERF4) contain an EAR-repression domain (Kazan, 2006). This repression seems to disappear after MeJA treatment, although the negative regulatory effect of WRKY26 on the cluster IV genes is enhanced.

We also examined the expression profiles of different MYC2-regulated TFs in this panel of mutants (Figure 9B). Examples of cross-regulation between MYC2-regulated TFs are the downregulation of *ERF4*, *TDR1*, *MYB34/ATR1*, and At1g06160 and the upregulation of *ERF1* and *WRKY26* by WRKY33, the downregulation of *EGL3*, *MYB34/ATR1*, and *MYB109* by WRKY26, and the upregulation of At1g06160 and *MYB109* by ERF2. Interestingly, basal transcript levels of *MYC2* seem to be repressed by ERF6, ERF11, and ZAT10. These expression profiles are illustrative of the regulatory complexities downstream of MYC2.

In an effort to better define the relative position of MYC2 within the JA signaling pathway, we wanted to know whether MYC2 is a primary JA response gene. The data shown in Figure 8 indicated that in the presence of CHX, the MeJA inducibility of MYC2 was abolished. Because de novo protein synthesis is not required for the induction of primary response genes by JA (van der Fits and Memelink, 2001; Pauw and Memelink, 2004), this observation indicates that, by definition, MYC2 is not a primary JA response gene. In additional experiments, we examined *MYC2*, *ERF1*, *PDF1.2*, and *VSP1* expression in wild-type plants treated with MeJA, CHX, or both. CHX treatment significantly induced *MYC2*, *ERF1*, and *VSP1* expression, and this induction was severalfold higher than that by MeJA (Figure 10A). By contrast, CHX treatment significantly suppressed *PDF1.2* expression. In the presence of the protein translation inhibitor CHX, the MeJA inducibility of *ERF1*, *PDF1.2*, and *VSP1* was abolished, as observed for MYC2 (Figure 10A). These experimental results are consistent with the model (Pauw and Memelink, 2004) proposing that, by definition, *MYC2*, *ERF1*, *PDF1.2*, and *VSP* are all secondary JA response genes requiring the synthesis of upstream regulators.

Next, we asked whether TF genes showing differential expression in *myc2/jin1* are direct or indirect targets of MYC2. We compared the expression of these TFs in CHX- and MeJA-treated *jin1-9* and *jin1-9/E35S:MYC2* based on the view that the existing levels of MYC2 should be sufficient to modulate the expression from primary target genes. By contrast, new protein synthesis would be required for the JA-dependent expression of secondary target genes (van der Fits and Memelink, 2001; Wang et al., 2005). Similar to *MYC2* and *ERF1*, the CHX treatment alone substantially induced all TF genes (data not shown), suggesting that the expression of these genes might be blocked by continuously synthesized repressors. Among the TF genes examined, *MYB34/ATR1*, *MYB75*, and *ZAT10* showed differences between CHX- and MeJA-treated plants of *jin1-9* and *jin1-9/E35S:MYC2* (Figure 10B). The MeJA-inducible differential expression of the remaining TFs in the mutant could not be observed in the presence of CHX, possibly due to the superinducibility of these genes by CHX treatment alone.

DISCUSSION

The results described here give MYC2 a central role within the JA signaling pathway in regulating diverse JA responses. Together with prior observations of MYC2 mediating crosstalk between JA–abscisic acid and JA–salicylic acid signaling, a novel role implicating MYC2 in auxin transport also indicates that MYC2 is a key junction point in a broader network involving multiple hormone signaling pathways.

Recently, JA signaling has been implicated in mediating the long-distance information transmission leading to a systemic immunity in *Arabidopsis* (Truman et al., 2007). Indeed, in the systemic tissue of plants challenged with avirulent bacterial or fungal pathogens, JA biosynthesis genes along with the genes involved in the synthesis of aromatic amino acids and glucosinolate and phenylpropanoid metabolism genes are induced (Schenk et al., 2003; Truman et al., 2007). Interestingly, the JA-mediated systemic defense against a virulent strain of *P. syringae* was compromised in the unchallenged leaves of the *myc2/jin1* mutant, which was locally treated with an avirulent strain (Truman et al., 2007), suggesting that MYC2 function is required for JA-mediated systemic resistance against bacterial pathogens.

The results presented here clearly show that MYC2 can indeed positively or negatively regulate many JA-dependent functions mentioned above. The differential effects of MYC2 on different JA responses might be due to the fact that precise coordination of these responses might be required for resource management and during adaptation to challenge by biotic and abiotic stress factors. For instance, although both pathogen and insect attacks stimulate JA biosynthesis, most changes in JA-responsive gene expression occur in an attacker-dependent manner (De Vos et al., 2005), suggesting that plants can divert limited resources in the best possible way. Therefore, one of the important functions of

Figure 9. (continued).

(A) Expression profiles of MYC2-regulated response/end point genes in mutant lines of MYC2-regulated TFs show clusters of coregulated genes. Samples were treated for 6 h with 0.1 μM MeJA (or mock controls). Data are means of three biological replicates (>20 pooled plants each) and are expressed as ratios of expression levels in the mutant lines to expression levels in the wild type. Clustering was done by complete linkage of Euclidian distances. Clusters of coregulated genes (I to IV) are shown in red at right, and the red line at left marks the cutoff distance used for the clustering.
(B) Expression profiles of MYC2-regulated TF genes in mutant lines of MYC2-regulated TFs illustrate cross-regulation between different TFs. Samples and data are as in **(A)**.

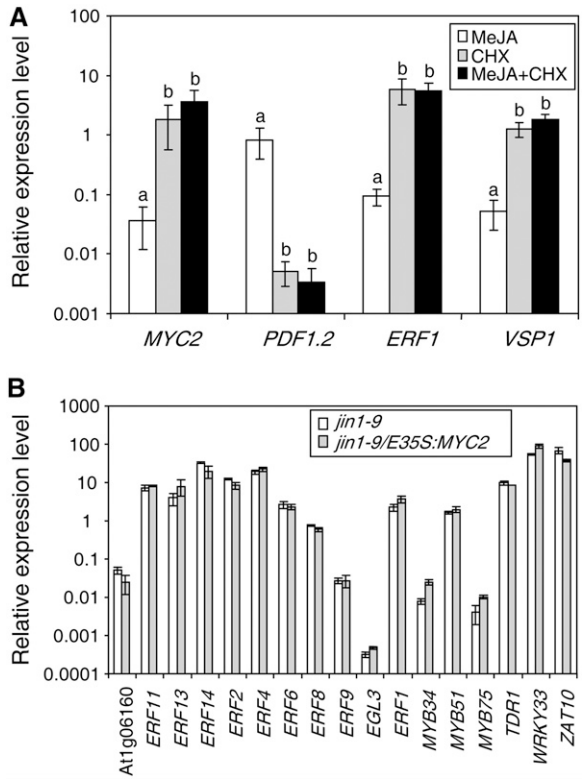

Figure 10. MYC2 Is a Secondary JA Response Gene.

(A) MeJA-, CHX-, and MeJA- and CHX-mediated expression of *MYC2*, *PDF1.2*, *ERF1*, and *VSP1*. Note that relative expression level on the *y* axis is given logarithmically. Data are means of three biological replicates. Error bars denote SE. Values annotated with different letters are significantly different ($P < 0.01$; Tukey's LSD).

(B) MYC2-modulated TF gene expression in CHX- and MeJA-treated plants of *jin1-9* and *jin1-9/E35S:MYC2*. Please note that relative expression level on the *y* axis is given logarithmically. See Figure 3A legend for details of Q-RT-PCR and Methods for details of treatments. Error bars denote SE.

MYC2 might be to coordinate JA-dependent defense responses by positively and negatively regulating JA-responsive insect and pathogen defense genes, respectively. Indeed, the *myc2/jin1* mutant shows increased resistance to fungal and bacterial pathogens but, as we have shown here, reduced resistance to an insect pest.

One would expect that *MYC2* expression itself should be tightly controlled at the transcriptional level during JA signaling for precise and rapid coordination of diverse JA-dependent responses. Indeed, Takahashi et al. (2007) proposed that there might be two separate JA-dependent pathways regulating *MYC2* expression. One of these pathways is dependent on MKK3 and MPK6 and negatively regulates *MYC2*, while the other is independent from these mitogen-activated protein kinase pathways and positively regulates *MYC2*. Our results presented here also indicate that MYC2 is capable of negatively regulating its own expression, possibly by binding directly to the G-box found its promoter. The fact that this negative autoregulation is consistently observed in multiple *jin1-9/E35S:MYC2* lines, while the opposite is not observed in *jin1-9*, suggests that this might be a mechanism operating when MYC2 levels reach a critical threshold, such as in plants simultaneously exposed to multiple biotic and/or abiotic stress conditions. Nevertheless, our findings, together with those by Takahashi et al. (2007), imply that both negative and positive regulation play roles in the control of *MYC2* expression and that this might be an important fine-tuning mechanism of the JA signaling pathway.

Our results from microarray experiments and subsequent functional analyses showed that in the absence of MeJA treatment, very few changes were observed in gene expression and phenotypic responses between the wild type, *myc2/jin1*, and *jin1-9/E35S:MYC2*. This suggests that additional factors induced and/or activated by MeJA might also be required for MYC2 action (Lorenzo et al., 2004). A plausible explanation, therefore, would be that JA activates MYC2 at the posttranscriptional level. Indeed, a reversible protein phosphorylation step is required for JA-mediated gene expression, as JA-dependent gene induction through this pathway was abolished in both *coi1* and *myc2/jin1* (Rojo et al., 1998). However, to date, the phosphorylation of MYC2 has not been demonstrated.

The large number of genes that showed some degree of significant MYC2 dependence for their JA-responsive expression might exclude the possibility that MYC2 directly controls the transcription of all of these genes. It is possible that MYC2 directly and indirectly regulates the JA-dependent transcription of a set of TFs, which in turn regulate the transcription of the secondary JA response genes. Several lines of evidence support this view. First, MYC2-regulated TFs are enriched for the presence of strong MYC2 binding sites in their promoter regions (see Supplemental Table 2 online). Furthermore, several JA-dependent TF genes show differential expression in the *jin1-9* mutant (see Supplemental Figure 1 online), and recent research suggests that some of these TFs can indeed regulate subsets of genes and phenotypes also regulated by MYC2 itself. For instance, comparison of our microarray data on *jin1-9* with that on *ERF1*-overexpressing plants (Lorenzo et al., 2003) showed that a number of genes negatively regulated by MYC2 were positively regulated by ERF1. This includes not only defense genes but also other functional categories such as Trp biosynthesis genes. Recent work has also shown that overexpression of the MYC2-repressed TF genes *ERF1*, *ERF2*, *TDR1*, *WRKY33*, and *ERF6* resulted in increased resistance to fungal pathogens such as *B. cinerea* and *F. oxysporum* (Berrocal-Lobo and Molina, 2004; Gutterson and Reuber, 2004; McGrath et al., 2005; Zheng et al., 2006b; C. Edgar and K. Kazan, unpublished data). Indeed, *myc2/jin1* shows increased resistance to all of these pathogens (Anderson et al., 2004; Lorenzo et al., 2004; Nickstadt et al., 2004; Laurie-Berry et al., 2006). Similarly, it was previously shown that overexpression of the MYC2-regulated TFs *MYB75/PAP1* and *EGL3* with reduced expression in the *myc2/jin1* mutant results in increased flavonoid biosynthesis (Tohge et al., 2005), suggesting that MYC2's effects on JA-mediated flavonoid metabolism are partially mediated by these TFs. The *myc2/jin1* mutant also shows increased resistance to *F. oxysporum* (Anderson et al., 2004), and as we found here, *ERF2* was upregulated in the mutant. *ERF2* encodes a positive regulator of JA-responsive defense genes, and overexpression of this TF leads to increased

F. oxysporum resistance in transgenic plants (McGrath et al., 2005). Moreover, our gene expression analyses in T-DNA lines of MYC2-modulated TFs suggest that these TFs might indeed regulate an overlapping subset of MYC2-modulated genes (Figures 9A and 9B). Further functional analyses of TFs directly or indirectly regulated by MYC2 should provide additional insights into the fine regulation of the different JA responses at the transcriptional level.

Some other effects of MYC2 on transcription could also occur as a result of MYC2-modulated changes in metabolite levels, such as altered flavonoid accumulation, changes in phytohormone balance (Nickstadt et al., 2004; Laurie-Berry et al., 2006), or altered redox status (Figure 5). For instance, our gene expression analyses (see Supplemental Figure 1 and Supplemental Table 1 online) suggest that MYC2 might modulate ET and JA levels by negatively and positively regulating the ET and JA biosynthesis genes *ACS6* and *AOC4*, respectively, during JA signaling.

Importantly, our results presented here show that MYC2 negatively regulates the Trp metabolic pathway during JA signaling. One class of Trp-derived secondary metabolites is the IGs. Our results are consistent with several reports that show that *Arabidopsis* IGs are elevated by treatment with MeJA, *Erwinia carotovora* elicitors, or *Phytium sylvaticum* and that an intact JA signaling cascade is required for their induction (Brader et al., 2001; Mikkelsen et al., 2003; Bednarek et al., 2005; Sasaki-Sekimoto et al., 2005). However, the transcriptional control of JA-mediated IG biosynthesis is not well known. Here, we showed that MYC2 is a negative regulator of JA-mediated IG biosynthesis, and again, this effect is likely to be at least partially mediated by the negative regulation of positive regulators of this pathway. Indeed, we found that the expression of *HIG1/MYB51*, encoding a positive regulator of this pathway, was increased in the *myc2/jin1* mutant. A recent report showed that HIG1/MYB51 activates IG biosynthesis genes such as *TSB1*, *ATR4/CYP83B1/SUR2*, and *ST5a* (Gigolashvili et al., 2007). Remarkably, both *HIG1/MYB51* and its downstream targets showed increased expression in the *myc2/jin1* mutant (Figure 2A). In addition, our expression analysis showed reduced expression of IG biosynthesis genes such as *ASB*, *TSA1*, *TSB2*, *IGPS*, *ST5a*, and *ATR4/CYP83B1/SUR2* in the JA-treated *myb51* mutant (Figure 9A).

Intact IGs and glucosinolates, in general, are thought to be nontoxic, but their breakdown products, isothiocyanates and nitriles, can be toxic (reviewed in Wittstock and Halkier, 2002; Grubb and Abel, 2006). Breakdown of glucosinolates to their nitrile derivatives is mediated through the ESP (Lambrix et al., 2001; Zabala et al., 2005). In the absence of ESP (Lambrix et al., 2001) or under conditions in which *ESP* expression is reduced (e.g., in a *myc2/jin1* mutant), glucosinolates spontaneously degrade to their respective isothiocyanate derivatives. *Arabidopsis* isothiocyanates have demonstrated in vitro (Olivier et al., 1999; Brader et al., 2001, 2006; Tierens et al., 2001) and in planta (Tierens et al., 2001; Brader et al., 2006) antimicrobial properties. Therefore, part of the enhanced disease resistance in *myc2/jin1* could be mediated by directing IG breakdown toward isothiocyanates. IGs are also involved in defense against certain insect pests. Despite increased levels of IGs, the *myc2/jin1* mutant showed reduced tolerance to *H. armigera*. However, we also observed reduced expression of wound and insect defense genes such as *VSP1*, *VSP2*, *At4g08870* (arginase), and *ADC2/SPE2* in the *jin1/myc2* mutant during JA signaling. This suggests that these insect defensive proteins might have a role in defense against *H. armigera*.

The potential of JA to induce auxin biosynthesis was originally proposed by Devoto et al. (2005). Here, we show experimentally that MeJA treatment can indeed increase IAA levels and that this could contribute to the MeJA-mediated growth regulation. This increase is probably due to the activation of both Trp-dependent and Trp-independent IAA biosynthesis (e.g., via ILR1). Plants overexpressing *ERF1* show both increased expression of genes encoding Trp biosynthetic enzymes and increased inhibition of root elongation by JA (Lorenzo et al., 2003), indicating that auxin homeostasis might also be altered in *ERF1*-overexpressing plants grown in the presence of exogenous JA. Interestingly, it was also shown that auxin increases the transcript levels of JA biosynthesis genes in *Arabidopsis* (Tiryaki and Staswick, 2002), suggesting that a positive feedback loop regulates these hormone levels. MeJA-mediated IAA synthesis may be critical for the proper regulation of plant growth and development under biotic stress. Indeed, a recent study in insect-attacked tobacco (*Nicotiana tabacum*) plants suggests that JA signaling suppresses regrowth and contributes to apical dominance, a role expected from auxin (Zavala and Baldwin, 2006). A similar role for auxin was also proposed for ET-mediated inhibition of root elongation (Rahman et al., 2001; Stepanova et al., 2005). ET inhibits root elongation through upregulation of the Trp biosynthesis genes *ASA1* and *ASB1*, which presumably leads to the accumulation of inhibitory levels of auxin in the root tip (Stepanova et al., 2005).

We also provided evidence that MYC2 is a positive regulator of enzymes and regulators involved in JA-mediated flavonoid biosynthesis. Flavonoids are recognized as endogenous regulators of auxin transport (Besseau et al., 2007, and references cited therein). Our experiments showed that *jin1-9/E35S:MYC2* roots were hypersensitive to the auxin transport inhibitors naringenin and TIBA. In addition to the previously known JA-insensitivity phenotype, the *myc2/jin1* roots exhibit increased resistance to the phytotoxin coronatine (Laurie-Berry et al., 2006). Both JA and coronatine induce flavonoid biosynthesis in wild-type plants, but this was compromised in the *myc2/jin1* mutant (Lorenzo et al., 2004; Laurie-Berry et al., 2006). Thus, we propose that the increased and reduced sensitivities of the *jin1-9/E35S:MYC2* and *myc2/jin1* roots, respectively, to exogenous JA might be due to altered flavonoid levels affecting auxin transport. A recent study by Zheng et al. (2006a) showed that, similar to JA, bestatin, an amino peptidase inhibitor, specifically activates the JA signaling pathway, induces *MYC2*, and inhibits root elongation in *Arabidopsis*. Remarkably, the *myc2/jin1* mutant shows reduced sensitivity to the bestatin-mediated inhibition of root elongation (Zheng et al., 2006a). Although the possible reason(s) for bestatin-mediated inhibition of the root elongation phenotype was not examined by Zheng et al. (2006a), previous studies showed that bestatin blocks auxin transport in a manner similar to flavonoids (Murphy et al., 2000).

The JA signaling pathway is known to modulate ozone-induced cell death in *Arabidopsis*, possibly by regulating ROS homeostasis. Most, if not all, JA signaling and biosynthetic mutants,

including *jar1*, *coi1*, *fad3*/*fad7*/*fad8*, *oji1*, and *opr3*, show increased ozone sensitivity (Kanna et al., 2003; Overmyer et al., 2003; Sasaki-Sekimoto et al., 2005). In addition, exogenous application of JA alleviates lesion formation in the ozone-sensitive *rcd1* mutant (Kanna et al., 2003; Overmyer et al., 2003). Here, we demonstrate that MYC2 is also a regulator of different MeJA-mediated antioxidant defenses. The decrease in oxidative stress tolerance of the *myc2*/*jin1* mutants under MeJA treatment is likely due to the combined effect of the reduced expression of genes associated with anthocyanin and tocopherol biosynthesis and ascorbate recycling.

Despite its quantitative effects on diverse JA-dependent processes, our results also indicate that MYC2 is not a primary JA response gene (see Pauw and Memelink, 2004, for further discussion). Indeed, recent studies showed that MYC2 acts downstream from COI1 and mitogen-activated protein kinase pathways in the JA signaling pathway (Lorenzo et al., 2004; Takahashi et al., 2007). Nevertheless, MYC2 probably acts relatively upstream within the secondary JA signaling cascade to affect the diverse JA-dependent phenotypes described here. Our results presented here also indicate that in the presence of CHX, MYC2 dependence could be observed in only a few TFs. The data presented in Figure 7D show that MYC2 binds strongly to the conserved sequence motifs found in the promoters of both *ERF1* and *ERF4*, suggesting that these genes are direct MYC2 targets. However, in the presence of CHX, differential expression of these genes by MeJA in *jin1-9* could not be observed. However, we found that almost all MYC2-modulated TF genes were superinduced by CHX alone, and as discussed by O'Connell et al. (2003), this has the potential to significantly mask the detection of MYC2 effects on downstream target genes. In addition, plant bHLH TFs are known to heterodimerize with either other bHLH- or MYB-type TFs prior to binding to target promoters. This might activate or repress transcription via the recruitment of histone acetyltransferase or histone deacetylase complexes to the target promoters. Therefore, the possibility exists that the synthesis and/or activity of a putative interacting protein or the recruitment of coactivator or corepressor complexes to MYC2 target promoters might also be influenced by CHX. Future experiments using chromatin immunoprecipitation followed by probing of genomic microarrays (ChIP-chip) (Lee et al., 2007) and independent validation should be useful for the large-scale identification of direct MYC2 targets.

In conclusion, our results reveal a number of novel functions for MYC2 in coordinating the responses in the JA signal transduction pathway. Future work on MYC2- and JA-regulated TFs could reveal additional information that might help us better understand the regulation of this important plant hormone signaling pathway as well as its interaction with other hormonal and developmental signaling pathways.

METHODS

Plant Growth Conditions, Chemical Treatments, and Pathogen Inoculations

Plant growth conditions and MeJA treatments (0.1 μM) were described previously (Schenk et al., 2000; Campbell et al., 2003; Anderson et al., 2004). All treatments were performed on soil-grown 4- to 5-week-old plants, unless stated otherwise. Plants were sprayed with a 50 μM methyl viologen (Sigma-Aldrich) solution (15 mL of solution was evenly sprayed over 90 plants). For CHX treatments, the aboveground tissues of 4-week-old soil-grown plants were submerged for 6 h in water containing 100 μM CHX in large tissue culture containers. When CHX was combined with MeJA (0.5 μM), the submerged plants were pretreated for 30 min with CHX before the addition of MeJA.

Arabidopsis Lines and Construction of Transgenic Lines

The following *Arabidopsis thaliana* lines have been described elsewhere: *jin1-9* and *jin1-10* (Anderson et al., 2004) and *max1-1* (N9564). Seeds for the mutant/T-DNA insertion lines were obtained from the ABRC or the Nottingham Arabidopsis Stock Centre. The location of the T-DNA insertion in the different TF genes was verified using a nested PCR approach (Alonso et al., 2003), and homozygous plants were used in all subsequent experiments. The mutant lines generated this way are as follows: *erf2* (SALK_136141), *erf6* (SALK_087356), *erf11* (SALK_516053), *wrky26* (SALK_563386), *wrky33* (SALK_006603), *myb51* (SALK_059771), *myb109* (SALK_068392), At1g33760 (SALK_569820), and *zat10* (SALK_054092).

Complementation of the *jin1-9* mutant background was done as follows. The coding region of *MYC2* (without the stop codon) was amplified from genomic DNA and ligated in pENTR/D-TOPO (Invitrogen). After sequence verification of correct amplification, the *MYC2* cDNA was recombined into the binary vector pCTAPi (Rohila et al., 2004) using the Gateway system (Invitrogen). Subsequent sequencing verified the correct in-frame cloning of *MYC2* fused to the CTAPi tandem affinity tag under the control of the enhanced cauliflower mosaic virus *35S* promoter. The construct was introduced into the *jin1-9* mutant background. Segregation analysis for BASTA resistance on T1 and T2 lines allowed for the selection of homozygous *jin1-9*/*E35S:MYC2* lines. These lines functionally complemented the *jin1-9* background for inhibition of root elongation by MeJA. Correct translation of the transgene was confirmed by protein gel blotting with the PAP conjugate (Sigma-Aldrich) reactive against the protein A domains of the CTAPi tag, as described before (Rivas et al., 2002).

Microarray Experiments and Data Analysis

The experimental factors of the microarray experiment were genotype (Col-0 versus *jin1-9*) and treatment (6 h of 0.1 μM MeJA versus mock controls), and for each genotype–treatment combination, three independent biological replicates were set up. In total, these yielded 12 samples (see Supplemental Methods online for more details). Each biological replicate (sample) consisted of the pooled material of 30 individual 4-week-old soil-grown plants from one tray (Col-0 and *jin1-9* were grown together in a randomized design per tray). Biological replicates (trays) were grown at different locations in the plant growth chamber and treated separately. For details of RNA processing, ATH1 GeneChip hybridization, and raw data collection, please see the Supplemental Methods online. All data analysis was done using the GeneSpring software package (version 7.2; Silicon Genetics). The probe-level intensities from the CEL files were normalized and summarized with the Robust Multi-Chip Average algorithm. The resulting expression measures were then normalized per gene to the median over the different chips. Because of the two-factor design of the experiment, the normalized expression values were analyzed by two-way ANOVA to determine whether either factor (genotype or treatment) had a significant effect on the expression level of a certain gene. The resulting P values ($P < 0.05$) were then subjected to multiple testing correction. This resulted in the substantial reduction of significant P values for the factor genotype, being indicative of the fact that in this experiment, the factor treatment had an overall greater effect on gene expression levels than the factor genotype. However, as we are primarily interested in the effect of genotype on gene expression levels, we

experimentally confirmed the expression of differentially expressed genes discussed in the text by Q-RT-PCR of the RNA samples used in the microarray experiment and of RNA samples from an independent time course experiment (see Results).

The lists of differentially expressed genes screened for significantly enriched Gene Ontology terms using DAVID (Dennis et al., 2003) are available in Supplemental Table 1 online.

Q-RT-PCR

Q-RT-PCR experiments were done as described elsewhere (McGrath et al., 2005). The sequences of the primer pairs have been published (Anderson et al., 2004; Czechowski et al., 2004; McGrath et al., 2005) or can be found in Supplemental Table 3 online.

DNA Binding Assays

A 1000-bp fragment of the *MYC2* coding sequence encompassing codons 285 to 623 was amplified from genomic DNA and cloned into the *Nhe*I-*Bam*HI–digested pTacLCELD6·His (Xue, 2005). The resulting construct encodes the last 338 amino acids of MYC2 (including the bHLH region) in-frame with the reporter protein CelD and a 6xHis tag. Correct amplification and cloning were verified by DNA sequencing. Determination of the consensus sequence of the MYC2 DNA binding motif and the relative binding affinity of these sites was done according to Xue (2005).

Insect Feeding Experiments

All experiments were performed on 5-week-old *Arabidopsis* plants. Plants were grown individually and in a completely randomized manner in soil in a large tissue culture container, and five neonate larvae (*Helicoverpa armigera*) were placed on each plant. The containers were sealed off with Miracloth to confine the larvae to the plant. After 6 d of feeding, the larval weight was determined on a precision balance.

Root Growth Inhibition Assays

Surface-sterilized *Arabidopsis* seeds were plated on half-strength Gamborg's B-5 basal medium or half-strength Murashige and Skoog medium (supplied with 5% sucrose and 0.7% Bacto Agar, pH 6.0). Media were supplemented with different concentrations of 5MT (Sigma-Aldrich; solubilized in 0.1 M NaOH), MeJA (Sigma-Aldrich; solubilized in absolute ethanol), naringenin (Sigma-Aldrich; solubilized in absolute ethanol), or TIBA (Sigma-Aldrich; solubilized in methanol). Plates were incubated under continuous light at 22°C, and seedlings were monitored between 7 and 10 d for root growth. Root lengths were measured using the ImageJ freeware package (http://rsb.info.nih.gov/ij/).

Measurements of Trp and Trp-Derived Metabolites

For soluble Trp, samples of 5-week-old *Arabidopsis* plants were frozen in liquid nitrogen and crushed with mortar and pestle. Approximately 100 mg of the crushed material was extracted at 4°C overnight in 20% methanol. Extracts were derivatized with the AccQ-Fluor reagent kit (Waters) and analyzed on an Acquity Ultra Performance liquid chromatograph (Waters).

For IGs and IAA, samples of 5-week-old *Arabidopsis* plants were prepared and analyzed as described before (Sarwar and Kirkegaard, 1998; Symons and Reid, 2003).

Microarray Data Deposition

Affymetrix data have been deposited in the ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) public repository under experiment number E-MEXP-883.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *MYC2* (At1g32640); *ERF2* (At5g47220); *ERF6* (At4g17490); *ERF11* (At1g28370); *ERF4* (At3g15210), *ERF13* (At2g44840); *ERF14* (At1g04370); *ERF8* (At1g53170); *ERF9* (At5g44210); *ERF1* (At3g23240); *WRKY26* (At5g07100), *WRKY33* (At2g38470); *MYB51* (At1g18570); *MYB109* (At3g55730), *ZAT10* (At1g27730); *GST6* (At1g02930); *CAD* (At4g34230); *ERF5* (At5g47230); *IGPS* (At2g04400); *CYP83B1/ATR4/RED1/SUR2* (At4g31500); *TSA1* (At3g54640); *PAD3* (At3g26830); *TSB2* (At4g27070); *ST5a* (At1g74100); *ACS6* (At4g11280); *PDF1.2* (At5g44420); *HEL/PR4* (At3g04720); *CHI/PR3* (At3g12500); *ADC2/SPE2* (At4g34710); *VSP1* (At5g24780); *MYB75/PAP1* (At1g56650); *TT19/GST12* (At5g17220); *PAL1* (At2g37040); *EGL3* (At1g63650); *ESP* (At1g54040); *ILR1* (At3g02875); *MYB34/ATR1* (At5g60890); *DHAR* (At1g19570); *AOC4* (At1g13280); *TDR1* (At3g23230); *VSP2* (At5g24770), *UGT79B1* (At5g54060); *APR3* (At4g21990); *APS3* (At4g14680); *MDHAR* (At3g09940); *TAT3* (At2g24850); *AACT* (At5g61160); At1g33760; At4g08870; At1g66100; At1g06160; and At3g28740.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Q-RT-PCR Expression Analysis of Selected MYC2-Regulated Genes in *jin1-9* versus Col-0 after MeJA (0.1 μM) or Mock Treatment.

Supplemental Table 1. List of *Arabidopsis* Genes That Are Significantly Affected in Their Expression by Genotype (Col-0 versus *jin1-9*), Treatment (mock versus 0.1 μM MeJA), or Genotype–Treatment Interaction.

Supplemental Table 2. List of *Arabidopsis* Genes with a Strong MYC2 Binding Site in the 3000-bp Upstream Region.

Supplemental Table 3. Sequences of the Primer Pairs Used for Q-RT-PCR.

Supplemental Methods. MIAME-Compliant Description of Microarray Experiments.

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NOTE ADDED IN PROOF

Nafisi et al. (2007) recently showed that CYP71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. MYC2 negatively regulates CYP71A13 (see Supplemental Table 1 online), providing additional evidence that MYC2 is a negative regulator of JA-dependent camalexin synthesis in *Arabidopsis*.

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